Suppressive Serum, Suppressor Lymphocytes, and Death from Burns

J. H. N. WOLFE, M.B., F.R.C.S.,*† I. SAPOROSCHETZ, A.B., A. E. YOUNG, M.Ch., F.R.C.S., N. E. O'CONNOR, M.D., J. A. MANNICK, M.D.

From the Department of Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts

Both suppressor lymphocytes and serum immunosuppressive factors have been found in patients who have had major thermal burns, and may inhibit host resistance to the bacteria invariably present in burn wounds. However, the relationship and clinical importance of these two manifestations of impaired immune reactivity are poorly understood. Eighteen patients (aged 20–84 years) with full thickness burns of varying severity have been studied, and the clinical course related to the presence of nonspecific immunosuppressive serum and circulating suppressor lymphocytes. Serum factors capable of suppressing the phytohemagglutinin (PHA) response of normal lymphocytes usually appeared early and were detected in 15 of the 18 patients at some time during the illness. Thirteen of these patients developed systemic infection. Depression of the PHA response of peripheral blood lymphocytes was much less common and was associated with a high mortality. Five of the eight patients with this finding died. No patients who did not have severe depression of the lymphocyte response to PHA died. Nonadherent leukocyte (NA leukocyte) populations exhibiting a depressed PHA response were capable of suppressing the PHA response of normal human lymphocytes and, therefore, contained suppressor cells.

SYSTEMIC INFECTION is the major cause of death and complications following a major thermal burn, and there is now extensive clinical[1,3] and experimental[8,18] evidence that host resistance to infection is impaired after a burn injury. Various facets of the immune response have been studied and shown to be deficient, but the complexity of the mechanisms involved is evident from the varied and sometimes conflicting reports.

Abnormal neutrophil function may play a role in the development of infection.[2] Alexander[3] found that burn patients who subsequently became infected had impaired neutrophil bactericidal activity to *Staphylococcus aureus*, but that the antibacterial function of neutrophils against *Escherichia coli* was similar in infected and uninfected patients. Since gram-negative infections usually occur during a more complicated course following a burn, other immunodeficiencies are probably important.

Patients anergic to intradermal injections of recall antigens have a worse prognosis,[16] and delayed hypersensitivity is considered to be a T-lymphocyte-mediated response. A profound impairment of the resistance to sepsis may thus be mediated through abnormal lymphocyte function.

It has been shown previously that a circulating immunosuppressive polypeptide(s), which inhibits phytohemagglutinin (PHA) proliferation of normal T lymphocytes, can be detected in the serum of severely burned patients.[4,6] The appearance of this immunosuppressive peptide(s) in the serum immediately precedes or coincides with the episode of sepsis and is not therefore considered to be a consequence of the infection. This conclusion is supported by the isolation of a similar peptide fraction from uninfected patients following accidental or operative trauma.[5,12] Several early reports have shown a normal or increased peripheral blood leukocyte response to PHA following a burn[7,10,11] which seemed to have little relationship to the effects of circulating suppressive factors in the serum, but recently Miller and Baker[13] reported the presence of circulating suppressor lymphocytes in burn patients. This report prompted the present study, in which the presence of serum immunosuppressive factors and the presence of circulating suppressor NA leukocytes were determined and related to the patients clinical course.

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† On leave of absence from the Department of Surgery, St. Thomas Hospital, London S.E. 1, England.

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Reprint requests: J. A. Mannick, M.D., Brigham and Women's Hospital, 75 Francis Street, Boston, Massachusetts 02115.

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Materials and Methods

Patients Studied

Eighteen patients (mean age: 44 years; range: 23–84) treated at the Peter Bent Brigham Hospital were studied. The initial therapy consisted of a Plasmanate and Ringer's lactate infusion for 48 hours, according to the patient's body weight and severity of the burn, and fluid replacement thereafter to cover the calculated loss. All patients were given tetanus toxoid and a 48-hour course of Benzyl penicillin. Their nutritional requirements for the anticipated hypermetabolic state were met according to the following formula: 40 kcal/kg body weight plus 40 kcal for each per cent burn per day. Their burn wound management consisted of daily Betadine baths, and twice daily Silvadine-impregnated fine mesh dressings. Escharotomies were performed when necessary, and early excisional therapy was reserved for hand burns only.

Culture samples were taken from the burn surfaces three times per week, and the patients were treated with the appropriate topical antibiotic ointment for five days if a dominant organism was found. Systemic antibodies were given if the patient had evidence of a chest or urinary tract infection or septicemia. On most of the patients, grafts were first performed between the twentieth and the twenty-fifth days, and the site was covered with prophylactic antibiotics for 48 hours (usually Oxycillin and Gentamycin).

The patients were categorized into two groups according to their chance of survival: less than 35% (Group 1), and greater than 35% chance of survival (Group 2). Their chance of survival was assessed from Peter Bent Brigham Hospital burn data by probit analysis which was based on the severity of the burn and the age of the patient. Group 2 was further subdivided into those patients who developed systemic infection (Group 2A) and those who did not (Group 2B). All patients developed surface wound infections.

Normal Controls

Control lymphocytes were obtained from normal fit volunteers (mean age: 30 years; range: 23–40) from among the laboratory personnel. Pooled normal serum was also obtained from fit volunteers, and the same pool was used throughout the study.

In Vitro *Assay of Immunosuppressive Activity*

Serum samples from the patients were tested for immunosuppressive activity *in vitro* by studying their ability to inhibit phytohemagglutinin (PHA) induced proliferation of normal human peripheral blood lymphocytes. Heparinized venous blood was obtained from the normal donors, and after gravity sedimentation of the erythrocytes for two hours at 20 C, the serum layer was placed on sterile wool columns and eluted with Eagles Minimal Essential Medium (MEM). After washing three times in MEM, the cells were counted and tested for viability by trypan blue dye exclusion. The procedure yielded a preparation of small lymphocytes, 95% or more pure and 95% or more viable. The micro method was used for testing lymphocyte stimulation. In the wells of flat bottomed microtest plates (Linbro Plastics) 2.5×10^5 lymphocytes were placed with 0.2 ml of MEM containing 1% glutamine, 5% fetal calf serum, 100 units of penicillin and 100 μg streptomycin per ml, and a range of stimulatory doses of purified PHA (2.5, 5, and 10 μg per ml). The optimum concentration was determined to be 2.5 μg PHA. Sera to be tested for immunosuppressive activity were added to the culture medium in 10% concentrations. Controls included cultures with no additions and those with 10% pooled normal serum. The same normal serum pool was used for all experiments. Microtest plates were incubated in a 5% CO_2 water-saturated environment at 37 C for 48 hours. ^{3}H-thymidine, 1 μc, was then added to each well. The cultures were processed 16–18 hours later by a Mash II Microharvester (Microbiological Associates) and counted in a Packard liquid scintillation counter. All determinations were performed in triplicate. Two lymphocyte donors were used on each occasion, and a mean of the results was obtained. The assay was performed on three separate occasions on each sample. Some wells in each experiment were used for a trypan blue viability determination of the cells incubated with or without the serum being tested. No cytotoxic sera were found in these experiments. Immunosuppression *in vitro* was calculated by the following formula:

$$\text{percentage suppression} = 1 - \frac{\text{cpm experimental wells with PHA} - \text{cpm control wells without PHA}}{\text{cpm control wells with PHA} - \text{cpm control wells without PHA}} \times 100$$

In these studies, 50% or more suppression of PHA stimulation by experimental serum when compared with control serum was considered significant.

Mitogenic Response of Lymphocytes

Peripheral venous blood samples from burn patients were obtained and the serum eluted through cotton wool columns as described above. Two normal controls were performed on each occasion. The NA leukocytes were then divided and either washed one, three, or six times before testing for mitogen stimulation.

The assay was performed at NA leukocyte concentrations of 1×10^5 and 5×10^4 in wells containing 0.2 ml of MEM containing 1% glutamine, 5% fetal calf serum, 100 units of penicillin, and 100 μg of streptomycin per ml. Purified PHA was then added at doses of 2.5 and 10 μg per ml, and the plates incubated and counted as described above. The optimum concentration was determined to be 2.5 μg PHA. The PHA response of patient lymphocytes was then expressed according to the following formula:

$$\text{percentage suppression} = 1 - \frac{(\text{cpm experimental wells} + \text{PHA}) - (\text{cpm experimental wells without PHA})}{(\text{cpm control wells} + \text{PHA}) - (\text{cpm control wells without PHA})} \times 100$$

Since there were two normal controls, the results were expressed as a mean of the two values obtained.

TABLE 1. *Clinical Outcome of Burn Patients*

	No. of Patients	Mean Per Cent Burn	Mean Age	Mortality	Systemic Infection
Group 1	5	81%	60.2	4 (80%)	4 (80%)
Group 2	13	39%	40.1	1 (7%)	9 (69%)

Evidence of circulating suppressor NA leukocytes was sought by adding together 5×10^4 cells from a patient and a normal control in the same well, with the media and incubation time used above, and measuring the PHA response.

The patient's NA leukocytes were tested with two separate populations of normal control NA leukocytes and the results expressed according to the following formula:

$$\text{percentage suppression} = 1 - \frac{\text{cpm patient's cells + control cells (1 or 2) with PHA} - \text{cpm patient's cells + control cells without PHA}}{\text{cpm (control cells 1 + control cells 2 + PHA)} - \text{cpm control cells 1 + control cells 2 without PHA}} \times 100$$

The final result was expressed as a mean of the two values obtained.

There was the problem of diluting normal responsive cells with unresponsive patient cells and thus achieving a false impression of suppressor cell activity. For evidence of suppressor cells it was considered necessary that the counts per minute following stimulation of the combined population of cells at 5×10^4 of each population (*i.e.*, 1×10^5 cells per well) were less than the counts per minute of 5×10^4 of the normal lymphocytes alone. This negated any dilutional effect and was expressed in the following formula:

$$\frac{(\text{cpm patient cells + normal cells with PHA at } 1 \times 10^5 \text{ cells per well}) - (\text{cpm patient cells + normal cells without PHA at } 1 \times 10^5 \text{ cells per well})}{(\text{cpm normal cells with PHA at } 5 \times 10^4 \text{ cells per well}) - (\text{cpm normal cells without PHA at } 5 \times 10^4 \text{ cells per well})}$$

Expressing the results in these ways only allowed for the most dramatic differences to be detected.[13] Incubation of two normal populations of cells (35 samples) led to a mean augmentation of the PHA response of 10% (two standard deviations: augmentation of 29% to suppression of 9%). An ability of patient NA leukocytes to suppress the normal lymphocyte mitogenic response by 40% was therefore highly significant. Unless the patient NA leukocytes were capable of producing this degree of suppression by both formula, suppressor cells were not considered to be present.

Results

The 18 patients studied were divided into two groups according to their chance of survival as assessed by probit analysis: Group 1 comprised those patients with a 35% or less chance of survival, and Group 2 consisted of those patients with a better than 35% chance of survival. Systemic infection and mortality rates in patients from these two groups are shown in Table 1.

Five patients were included in Group 1, and four of these died (mean percentage burn = 81%, mean chance of survival = 14%) and one survived (Table 1). The other 13 patients formed Group 2. The only death in this group was a 54-year-old man who died of a pulmonary embolus on the eighth day after injury.

Episodes of systemic infection with multiple gram-positive and gram-negative organisms occurred in all the Group 1 patients with the exception of a 75-year-old man who died with a myocardial infarct and renal failure within 36 hours of admission.

Since a failure of the immune response may have

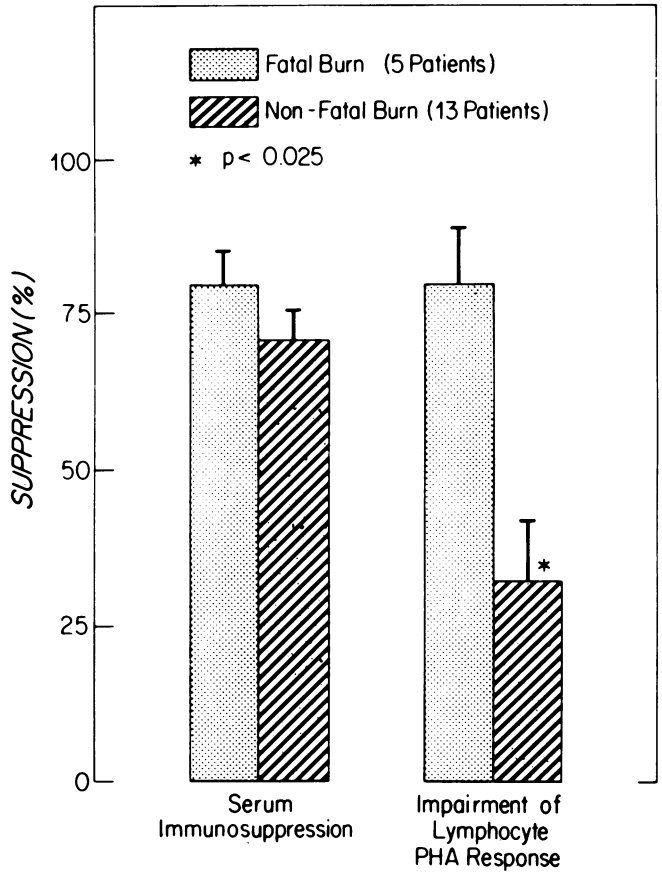

FIG. 1. Mean serum immunosuppression (±SD) and impaired lymphocyte response in fatal and nonfatal burns. * Lymphocyte response to PHA significantly less ($p < 0.025$) in fatally burned patients.

played a role in these systemic infections, Group 2 patients were then classified as infected (Group 2A) or uninfected (Group 2B). Three patients had septicemia with positive blood cultures for multiple gram-positive and gram-negative organisms. Four of them had systemic *Staphylococcal aureus* infections.

The immunologic response of the patients could be studied in the light of the marked difference in the clinical outcome between Group 1 and Group 2 patients. In Figure 1 the greatest serum immunosuppression and impairment of lymphocyte response to PHA recorded in each patient are compared in fatal and nonfatal burns. Four of the deaths were due to infective complications, and one was due to pulmonary embolism. The serum immunosuppression was very similar in these two groups, but there was a statistically significant difference in the response of the lymphocytes to PHA ($p < 0.025$, using the Student's t-test).

Serum Immunosuppressive Factors

Sixty-three samples (from 18 patients) were measured (Fig. 2). Only the sample with the greatest immunosuppressive activity, usually taken during the second or third week following the burn, was used in each patient to avoid undue weight being given to samples taken during the recovery phase of the illness.

The only patient in Group 1 who did not have suppressive serum survived; the other four died and had very severe serum immunosuppression (mean of immunosuppressive activity of patients who died = 83.25%). This includes the patient who had no evidence of infection but died soon after admission.

Surprisingly, most patients in Group 2 also had severe serum immunosuppression at some stage of their illness (mean greatest immunosuppressive activity in Group 2 patients = 74.7%). Even when a comparison was made between the systemically infected Group 2A patients and the Group 2B patients who did not have systemic infection despite their wound infections, there was only a minimal difference in the serum immunosuppression (mean 75% serum immunosuppression in Group 2A and 72% in Group 2B).

It therefore appears that most burn patients develop serum immunosuppression during the course of their illness, and that it is not a good measure of clinical outcome, although more severely burned patients tend to have more serum immunosuppression.

Suppression of Lymphocyte PHA Response

On 57 occasions (in 18 patients) the response of the patient's lymphocytes to PHA stimulation was tested.

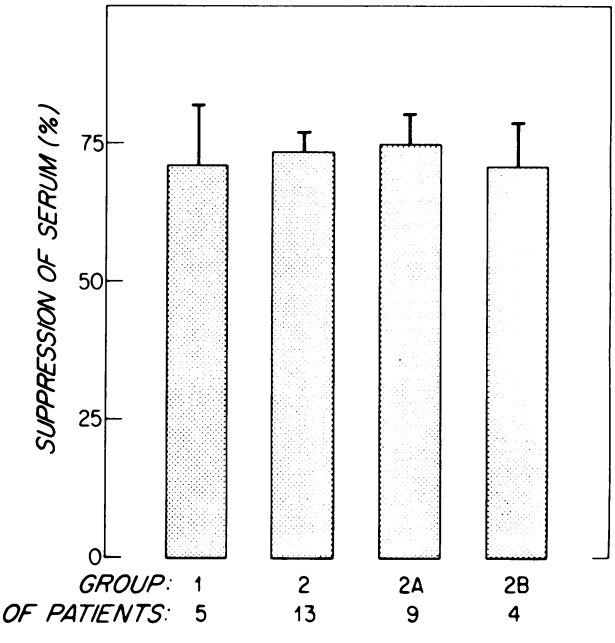

FIG. 2. Mean serum immunosuppression (±SD) in burn patients. * Lymphocyte PHA response significantly better ($p < 0.05$) in uninfected Group 2B patients than infected Group 2A and Group 1 patients.

A reduction in PHA stimulation correlated better with the severity of burn than the serum immunosuppression (see Figure 2). The mean greatest suppression of PHA response in the Group 1 patients was 60%, but in Group 2 it was 40% (Fig. 3). Furthermore, the uninfected Group 2B patients had only a 14% mean suppression of their PHA response as compared with 52% suppression in the infected Group 2A patients. The Group 2B patients had a statistically significant greater response to PHA than the Group 1 and Group 2A patients ($p < 0.05$, Student's t-test). This improved response reflects not only a less severe burn injury but also a lack of subsequent infective complications.

Presence of Suppressor NA Leukocytes

On 32 occasions (in 7 patients) the circulating peripheral NA leukocytes were tested for the presence of suppressor cells (Fig. 4).

The combined incubation of two normal populations of NA leukocytes, each at a concentration of 5×10^4 per well, as compared with a single population of normal cells at a concentration of 1×10^5 cells per well leads to a mean augmentation of the PHA response of 10% (two standard deviations: augmentation 29% to suppression 9%). Two populations of normal NA leukocytes never produced a suppression of more than 25%.

One patient had suppressor NA leukocytes capable of suppressing normal cell response by more than 50%, and she died of overwhelming systemic infection (see Figure 5). Only slightly lesser degrees of suppressor cell activity (greater than 40% suppression) were present in another two patients. A 32-year-old woman with a 40% burn, who might have been expected to have a relatively uncomplicated illness, had a protracted hospital admission (65 days) and developed gram-negative septicemia and *Candida albicans* septicemia subsequent to the appearance of circulating suppressor NA leukocytes. The second patient was a 61-year-old man with a 35% burn who also had a protracted hospital admission (89 days) and had intermittent systemic infections due to gram-negative and gram-positive organisms.

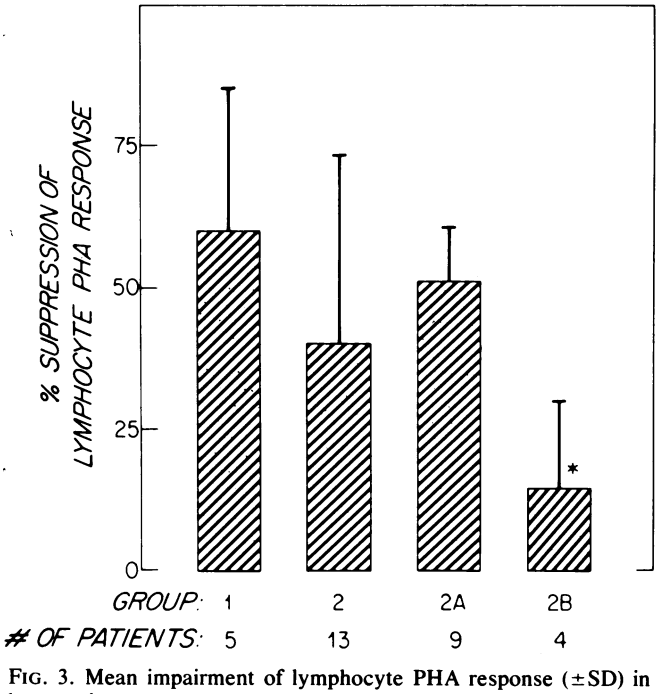

FIG. 3. Mean impairment of lymphocyte PHA response (±SD) in burn patients.

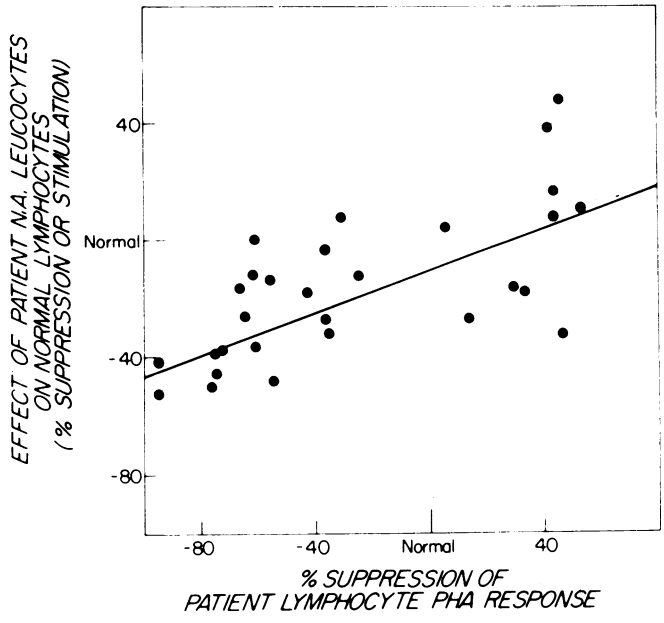

FIG. 4. Correlation of suppressor lymphocytes with suppression of lymphocyte PHA response ($r = 0.714$, $p < 0.005$).

The presence of circulating suppressor NA leukocytes correlated with the depression of the lymphocyte response to PHA ($r = 0.71$, $p < 0.005$) as shown in Figure 4. Depression of PHA mitogenesis was more pronounced than the suppressor cell activity of these same cell populations.

Failure to Improve Lymphocyte Response by Repeated Washings

It was possible that serum suppressive factors loosely adherent to the lymphocytes were the cause of the cellular suppression. The PHA response was therefore tested in 27 samples of patient's lymphocytes after a single cell washing and again after six washings. There was an improvement of more than 50% in the counts per minute after six washings on five occasions (18% of the samples), and there was an overall improvement in the counts per minute of 13% after six washes.

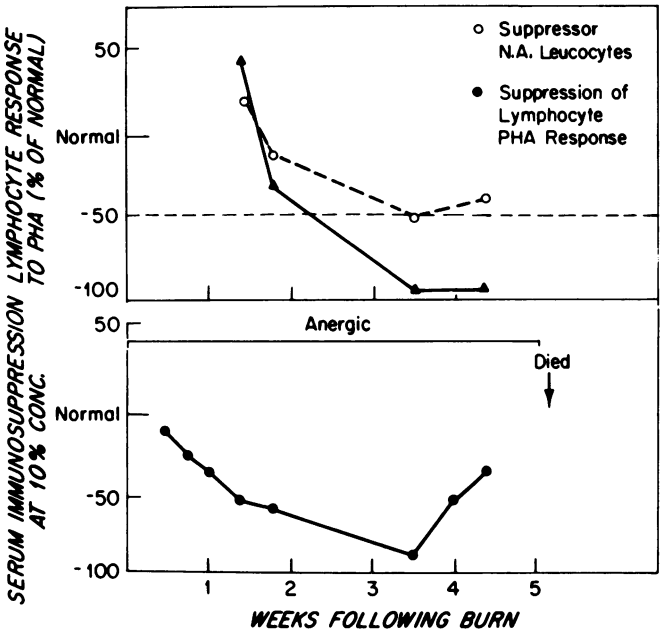

FIG. 5. Course of serum suppression and suppression of lymphocyte response to PHA following fatal burn (patient age 42, 80% third-degree burn).

This overall difference was not significant (mean cpm after one washing: 39905; mean cpm after six washings: 45114) but in 18% of patients the improvement in response was significant. In a further 11 samples, there was no difference in the response of cells following three and six washings (mean cpm after three washings: 39209; mean cpm after six washings: 34774). The results presented in this paper were derived from cells washed three times, and are not considered to be affected by loosely adherent serum suppressive factors which might affect results following a single cell washing.

Serial Serum and Lymphocyte Studies

In 12 patients, serial studies of the serum immunosuppressive activity and NA leukocyte function were performed. Serum immunosuppressive factors usually developed during the first week following the burn, and prior to the development of systemic infection. When the PHA response of the lymphocytes was impaired, it occurred at the same time as serum suppression or a few days later, and was associated with suppressor NA leukocyte activity. Figures 5 and 6 illustrate these events in two patients.

Patient A (Fig. 5) was a 42-year-old woman with an 80% third-degree burn, who died 36 days after admission of overwhelming infection with multiple organisms. The three initial serum samples were not suppressive, and 50% suppression developed after one week. The serum then remained suppressive until the last sample taken the day before death.

For the initial ten days, there were inadequate numbers of peripheral lymphocytes to test their function. On the first sample measured, the circulating lymphocytes had an augmented PHA response, *i.e.*, at a time when the serum was already suppressive. The PHA response subsequently deteriorated until the patient's death, with an associated development of suppressor NA leukocytes.

Patient B (Fig. 6) was a 23-year-old man who also had a severe burn (65% third degree), but his immunologic response to the injury and clinical outcome were quite different. He developed serum immunosuppression within two days of the injury, and this reached its nadir four weeks later before recovering. As in the first patient, the lymphocytes had an initially augmented response to PHA, and although some suppression of the response appeared intermittently in the subsequent weeks, this was never sustained, and

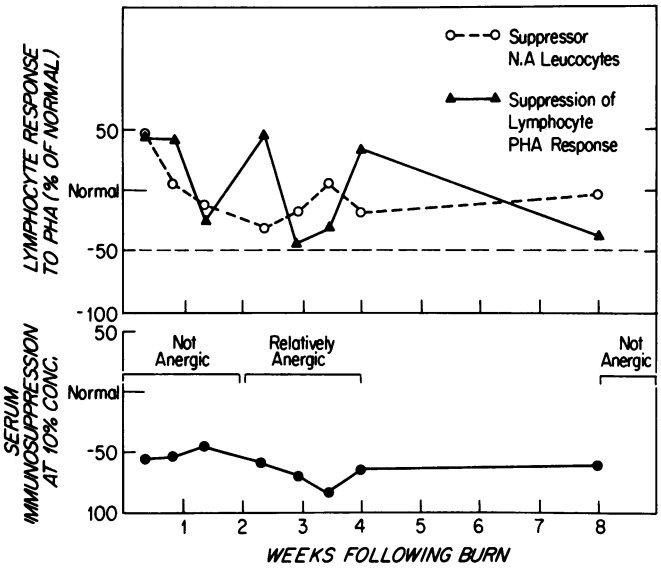

FIG. 6. Course of serum suppression and suppression of lymphocyte response to PHA following nonfatal burn (patient age 23, 65% third-degree burn).

significant suppressor NA leukocyte activity never developed.

Discussion

Bacteria are universally present in burn wounds and their invasion of the bloodstream is the commonest cause of complications and death in these patients. The presence of bacteremia is related to the number of bacteria present (which in turn is closely related to the severity of the burn), and the ability of the host to resist infection. Considerable advances have been made in the care of burn wounds and in the control of infection, but the problems of impaired host resistance have not been overcome since the delicate balance of immunoregulation is still poorly understood. Many aspects of the immune system have been studied and shown to be abnormal, and these can be divided broadly into the neutrophil function, the opsonic factors in the serum, and the interaction between lymphocytes and macrophages.

The neutrophil function is abnormal following a severe burn.[3,20] One report stated that the ingestion of bacteria remained normal but the intracellular killing was impaired.[2] This was only true, however, of *S. aureus*, and the same neutrophils were fully active against *E. coli*.[3] In that case the neutrophil chemotactic response also failed to correlate with the onset of infection. There is disagreement in the literature whether neutrophil chemotaxis correlates with survival.[9,22] Since it is well recognized that gram-negative septicemia is usually the harbinger of a deteriorating clinical condition and normal neutrophil function against *E. coli* has been demonstrated in burn patients, the major component in the breakdown of host resistance may not be neutrophil dysfunction.

Anergic patients have a worse prognosis than those who have a response to intradermal injection of recall antigens, and this response is considered to be T-cell mediated.[18] Several reports of normal or augmented PHA responses of burned patient's lymphocytes[3,7,10,11] diverted attention away from studying these cells. Patients were not, however, studied on repeated occasions. Time-related changes may have been missed, and the patients were not usually categorized according to the severity of the injury.[7] Recently Miller and Baker[13] studied burn patients who developed suppressor T cells, and Ninnemann[15] described suppressor cells that required B-cell activation in burned patients.

This study has therefore concentrated on the detection of serum suppression of lymphocyte activation and its relationship to abnormal lymphocyte function. The categorization of the patients by probit analysis correlated very closely with mortality, enabling the immunologic tests to be reviewed in relation to clinical outcome.

The presence of immunosuppressive peptide(s) in the serum of anergic, traumatized, and burned patients has previously been shown to correlate with clinical outcome.[6,12] In this study, however, serum immunosuppression was a ubiquitious event in burned patients and of little value as a clinical discriminant. Serum immunosuppressive activity was almost identical in the high mortality group Group 1 patients and the Group 2 patients. Surprisingly, the Group 2B patients who did not develop septicemia had a similar degree of serum immunosuppression at some time during their illness as the patients who developed systemic infections. A similar observation was made by Alexander et al.[3]

Without the benefit of serial samples from an individual patient, these results might suggest that serum immunosuppression is of little significance. However, serial samples revealed that serum immunosupression preceded suppression of lymphocyte PHA response and the development of circulating suppressor NA leukocytes and suggests that the serum factor may initiate the events leading to the production of suppressor lymphocytes. This hypothesis is supported by some recent experiments from this laboratory in which serum fractions from burn patients were shown to induce splenic suppressor cells when injected into mice (Wolfe, Mannick et al., unpublished data).

The suppression of the circulating lymphocyte mitogenic response to PHA was a less frequent event and a good discriminant of clinical outcome. Miller and Baker[13] came to the same conclusion, and this association was also alluded to earlier by Munster.[14] In the present study, there was statistically significant difference between the suppression of the PHA response of the uninfected Group 2B patients and the infected Group 1 and 2A patients so that a suppressed PHA response related not only to the mortality but also to a failure to resist systemic infection.

The serial studies show that an initial augmentation of the lymphocyte PHA response is common, and may be present intermittently throughout the illness in patients who do not develop severe immunosuppression (Fig. 6). This confirms the previous reports of an augmented response following injury, but by taking subsequent samples it was possible to show the development of an impaired response in some patients. This impaired response, when associated with circulating suppressor NA leukocytes, is associated with a breakdown of immunocompetence that may be fatal.

However, the interpretation of these results must be tempered by several considerations. First, the cells were usually fractionated on cotton wool, not nylon wool columns, since this gave us a better cell yield in patients who were often lymphopenic. The suppressor cell is a nonadherent cell, but this is not synonymous with being a lymphocyte. The term NA leukocyte has

therefore been used throughout. On the occasions that nylon wool columns were used, similar results were obtained, which provide evidence that the suppressor NA leukocytes were T cells. However, the presence of B cells was not excluded, and according to Ninnemann,[15] the T-suppressor cell in burn patients may be dependent on a B cell for its activity.

Second, no control levels of lymphocyte response can be obtained in a burned patient. The initial sample, before there was any evidence of suppression, was used as a control by Miller and Baker.[13] This would have been inappropriate in the present study since the initial response was often augmented, and if used as a control the subsequent suppression would have been falsely increased. The burn patients have therefore been compared to simultaneously studied normal volunteers.

Third, although the background counts were always low (less than 2,000 cpm), they were slightly higher in the patients than in the normal controls on many occasions, suggesting an unstimulated proliferation of the patient's cells. This phenomenon has previously been reported.[7,8,10,11,19] When the counts per minute following PHA stimulation were similar in the patient and normal control, results derived from the stimulation index (cpm following PHA stimulation ÷ background cpm) would have given a result suggesting suppression of the PHA response. For this reason, the reported results were obtained from counts per minute and not from the stimulation index.

Finally, it was shown that the presence of loosely bound serum suppressive factors may affect results following a single washing of peripheral blood lymphocytes, but that further washing removed this factor(s) and lymphocyte blastogenesis remained similar after 3 and 6 washings. From this it was concluded that the lymphocytes themselves were suppressed and that the suppression measured after three washings was not due to loosely bound serum factors.

The results of this study suggest that the trauma of the burn injury results in the production of serum immunosuppressive factor(s) in most patients. The subsequent development of suppressor NA leukocytes is an event associated with systemic infection and a high mortality, suggesting a breakdown in immunocompetence. The apparently self-destructive nature of this mechanism needs explanation but remains conjectural at the present time. The regulatory effect of suppressor NA leukocytes on immunoreactivity is well recognized, and following a burn the loss of the skin barrier presumably leads to an enormous antigenic bombardment of the patient's immune system. It is possible that the unregulated response to these antigens could be harmful to the host and that the suppressor cell mechanisms control this response.[17] In severely burned patients, the antigenic stimulus may be so great, and the regulatory suppressor cells so active, that immunodeficiency is the result. It is possible that the suppressive serum factor(s) play a role in triggering the production of suppressor NA leukocytes and its source and chemical structure are therefore of great interest.

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